Recommendations for Consideration in the Research Use

of Inflammatory Agents[1]

Complete Freund's Adjuvant (CFA)

CFA, a water-in-oil emulsion containing heat-killed mycobacteria or mycobacterial cell wall components, is regarded as an effective means of potentiating cellular and humoral antibody response to injected immunogens. Adjuvant activity is a result of sustained release of antigen from the oily deposit and stimulation of a local innate immune response resulting in enhanced adaptive immunity. An essential component of this response is an inflammatory reaction at the site of antigen deposition resulting from an influx of leukocytes and their interaction with antigen. The use of CFA is an important biologic resource for investigators, which should be used responsibly and with care to avoid or minimize the adverse effects of excessive inflammation. CFA may result in local inflammation and granulomatous reactions at the site of injection. CFA used improperly or excessively can cause side effects such as chronic inflammation, skin ulceration, or draining sinuses. Other complications observed following CFA use are, diffuse systemic granulomas secondary to migration of the oil emulsion, adjuvant related arthritis, and chronic wasting disease. The following guidelines are directed toward the elimination or minimization of complications secondary to immunization with CFA.

Utilization of: a) sterile technique in the preparation of antigen-adjuvant emulsions; b) aseptic preparation of the injection site; c) appropriate injection technique; d) appropriate routes and sites of administration; e) adequate separation of injection sites; and f) use of smaller volumes at each injection site, have all proven efficacious in the elimination of post immunization complications. The mycobacteria in CFA should first be resuspended by vortexing or shaking. Removal of the CFA from the ampule or vial should be accomplished using sterile technique. Although approaches may vary, one part or less of CFA to one part antigen (v/v) has been recommended (1). Although formulations of CFA containing 0.5 mg/ml mycobacterial concentration are commercially available and have been used successfully by many, concentrations of $\leq$ 0.1 mg/ml have been recommended to minimize the inflammation and necrosis observed with higher concentrations (2). Use of greater concentrations than commercially available are not recommended unless scientifically justified and approved by the institutional ACUC. In addition, use of preparations containing disrupted mycobacterial cells rather than whole, intact bacilli may prove efficacious because of the inability of the later to be histologically distinguished from live acid-fast cells. Antigen preparations should also be sterile and, ideally, isotonic and pH neutral. Antigens in polyacrylamide gels should be separated whenever possible or the amount of polyacrylamide gel should be reduced by careful trimming to minimize the amount of secondary inflammation/irritation from polyacrylamide (See Below).

Prior to immunization, the injection site should be clipped and surgically scrubbed to minimize the chance of bacterial contamination Experience has demonstrated that using injection volumes and sites appropriate for the species, size of the animal and experimental goal (Table 1) have produced favorable results while minimizing undesirable side effects (3, 4). Some routes of injection may potentially be less disruptive to the animal than other routes (e.g. subcutaneous injection vs. foot-pad administration, etc.). Whenever possible the least invasive methodology required to accomplish the experimental goal should be utilized. In addition, to the route of administration the site of injection should be chosen with care to avoid areas, which may compromise the normal movement or handling of the animal (e.g. intradermal injections in the scruff of the neck of a rabbit, etc.). If raising hyperimmune serum, CFA is usually only necessary for the initial immunization, while incomplete Freund's adjuvant, without mycobacterium, is the adjuvant of choice for subsequent immunizations

Non-inflammatory adjuvants or adjuvants known to produce less intensive inflammatory responses should be considered. These include TiterMax®, Ribi Adjuvant System (RAS), aluminum compounds, subcutaneously implanted chambers (5) and others. Experience has demonstrated that in many situations these alternatives to CFA are capable of eliciting a sufficient humoral antibody response. Information on alternatives to adjuvants and antibody production is available at Resources for Adjuvants and Antibody production: Comparisons and Alternative Technologies, Resource Series No. 3, March 1997, Animal Welfare Information Center (AWIC), NAL, USDA, 10301 Baltimore Boulevard, Beltsville, MD 20705 and also on-line at: http://www.nal.usda.gov/awic/pubs/antibody/.

Footpad Immunization

Utilizing the footpad for immunization of small rodents may be necessary in particular studies where isolating a draining lymph node, as a primary action site, is required. The well-being of subject animals should be addressed by procedures such as controlling the quantity of adjuvant instilled in the footpad, the use of only one foot per experimental animal, and housing on soft bedding rather than screens. Lacking evidence indicating a specific requirement, this technique should not be used for routine immunization of rodents. If scientific justification is provided, the recommended maximum footpad injection volumes are 0.01-0.05 in mice and 0.10 ml for rats (1). Rabbits should not be immunized in their footpads because they do not have a true footpad.

Peritoneal Exudate

The production of rodent peritoneal exudate by the intraperitoneal administration of antigen and adjuvant is a widely recognized scientific procedure for obtaining high titered reagent. Undesirable side effects of painful abdominal distention and the

resulting distress can be avoided by daily monitoring and relieving of ascites pressure, or terminating the procedure Intraperitoneal injections should normally be limited to less than 0.2 ml in mice (6).

Post-injection Observations and Treatments

All animals immunized with CFA or an alternative product should be monitored daily for adverse reactions, pain, or distress. These observations should continue daily until all lesions have healed. Supportive therapy may include topical cleansing, antibiotics, and use of an analgesic. Although analgesics are not routinely required, the use of narcotic agonists or mixed agonists-antagonists, or other species appropriate agents should be used if overt pain or distress is observed. Steroidal or non-steroidal anti-inflammatory agents must be used with caution due to their direct impact on the immunological process.

Personnel Safety

Handling of CFA can be an occupational hazard to laboratory personnel. Reports of accidental needle punctures in humans have been associated with clinical pain, inflammatory lesions, and abscess formation in tuberculin-positive individuals. Tuberculin-negative individuals tested positive in subsequent tuberculin tests after accidental CFA exposure (7). Safety glasses should be worn to avoid accidental splashing of CFA in the eyes.

Other Considerations

Scientists preparing antigens for in vivo administration in animals in conjunction with adjuvants should be aware of the potential presence of substances and other characteristics of the injectate, which may have additive inflammatory effects. Judicious use of adjuvant may be abrogated by failure to consider sterility of preparations, excessive vehicle pH, or the presence of by-products of purification such as polyacrylamide gel. Care should be taken to consider and eliminate additional inflammatory stimuli when possible.

Table 1. Recommended Volume of CFA-Antigen Emulsion (CFA-AE) per Site and Route of Administration

SPECIES	SUBCUTANEOUS	INTRADERMAL	INTRAPERITONEAL	FOOTPAD	INTRAMUSCULAR
Mouse	≤0.1 ml	*	≤0.2 ml	≤0.05 ml**	≤0.05**
Rat	≤0.1 ml	≤ 0.05 ml	≤0.5 ml	≤0.1 ml**	≤0.1**
Rabbit	≤0.25 ml	≤0.05 ml	*	*	≤0.25***
Goat/ Sheep	≤1.0 ml	≤0.1 ml	*	NA	≤0.5 ml

* Not recommended
** Only When Justified
*** Only One Limb Recommended Without Justification
NA Not applicable

Reference:

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